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Expression of CYP 4A ω-hydroxylase and formation of 20-hydroxyeicosatetreanoic acid (20-HETE) in cultured rat brain astrocytes

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ABSTRACT

Astrocytes secrete vasodilator and vasoconstrictor factors via end feet processes, altering blood flow to meet neuronal metabolic demand. Compared to what is known about the ability of astrocytes to release factors that dilate local cerebral vasculature, very little is known regarding the source and identity of astrocyte derived constricting factors. The present study investigated if astrocytes express CYP 4A ω-hydroxylase and metabolize arachidonic acid (AA) to 20-hydroxyeicotetraenoic acid (20-HETE) that regulates K_{Ca} channel activity in astrocytes and cerebral arterial myocyte contractility. Here we report that cultured astrocytes express CYP 4A2/3 ω-hydroxylase mRNA and CYP 4A protein and produce 20-HETE and the CYP epoxygenase metabolites epoxyeicosatrienoic acids (EETs) when incubated with AA. The production of 20-HETE and EETs was enhanced following stimulation of metabotropic glutamate receptors (mGluR) on the astrocytes. Exogenous application of 20-HETE attenuated, whereas inhibition of 20-HETE production with HET-0016 increased the open state probabilities (NPo) of 71 pS and 161 pS K_{Ca} single-channel currents recorded from astrocytes. Exposure of isolated cerebral arterial myocytes to conditioned media from cultured astrocytes caused shortening of the length of freshly isolated cerebral arterial myocytes that was not evident following inhibition of astrocyte 20-HETE synthesis and action. These findings suggest that astrocytes not only release vasodilator EETs in response to mGluR stimulation but also synthetize and release the cerebral arterial myocyte constrictor 20-HETE that also functions as an endogenous inhibitor of the activity of two types of K_{Ca} channel currents found in astrocytes.

1. Introduction

Astrocytes are the most abundant cell types in the brain and are known to provide structural and metabolic support to neurons. Astrocytes send foot processes to neurons and adjacent cerebral microvessels, and communicate with each other through gap junctions. They couple neuronal activity to local blood flow as a component of the neurovascular unit (NVU) [1,2,3]. A variety of membrane-derived lipids is emerging as important regulators of cerebrovascular function under physiological and pathological conditions [4–12]. Enzymes of the cytochrome P450 (CYP) gene family are expressed in different cell types in the brain and catalyze conversion of AA released by phospholipase A2 to a variety of fatty acid signaling molecules [5–7,12–16]. We have previously reported that

Abbreviations: 20-HETE, 20-hydroxyeicosatetraenoic acid; CYP 4A enzyme, cytochrome P450 4A ω-hydroxylase; EETs, epoxyeicosatrienoic acids; AA, arachidonic acid; HET0016, ((*N*-hydroxy-*N*′-(4-butyl-2-methylphenyl)formamidine; CBF, cerebral blood flow; LC–ESI–MS, Liquid chromatographic–electrospray ionization–mass spectrometric; LC–MS, liquid chromatography–mass spectroscopy; K_{Ca}, calcium-activated K^+ channel; NPo, channel open state probability; mGluR, metabotropic glutamate receptor; GFAP, glial fibrillary acidic protein; SAH, subarachnoid hemorrhage.

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brain astrocytes express the CYP 2C11 and 4X1 epoxygenases, and the CYP 4F5 and 4F6 ω-hydroxylases in addition to the presence of cyclooxygenases (COX) [5–7,17,18]. These enzymes metabolize AA to epoxyeicosatrienoic acids (EETs) and prostaglandins (PGs) [6,7,10,18–20]. The EETs can activate Ca^{2+}-activated K^+ channel (K_{Ca}) channel currents in adjacent arteriolar muscle cells to hyperpolarize the membrane potential and elicit cerebral vasodilation [21,22]. Since the production and release of EETs by astrocytes is increased in response to elevated neuronal activity, the EETs are now regarded as one of the astrocyte-derived vasodilator factors along with NO, adenosine, prostaglandins and K^+ that mediate functional hyperemia in the brain [21–25]. EETs also increase the activity of two types of K_{Ca} channel currents in astrocytes and initiate signaling cascades that regulate astrocyte function [26]. In addition to the CYP epoxygenases catalyzing formation of EETs from AA, earlier studies from our laboratory identified the expression of CYP ω-hydroxylase isoforms that catalyze the ω-hydroxylation of AA to 20-hydroxeicosatetraenoic acid (20-HETE) in different cell types in the brain [4,6–8,11,27]. The CYP 4A11, CYP 4A22, CYP 4F2 and CYP 4F3 isoforms are all expressed in man and have been reported to catalyze the formation of 20-HETE from arachidonic acid [28]. CYP4A10, 4A12a, 4A12b and 4A14 are the isoforms expressed in mice. CYP4A12a and b convert arachidonic acid to 20-HETE, while the activity of the 4A10 and 4A14 isoforms are very low [29]. The corresponding isoforms expressed in the rat are CYP4A1, CYP4A2, CYP4A3 and CYP4A8. Of these, CYP4A2 and 4A3 are most avidly expressed and all 4 isoforms metabolize arachidonic acid to 20-HETE [28]. 20-HETE is a potent vasoconstrictor that is formed in cerebral arterial muscle cells and acts through PKC-dependent inhibition of K_{Ca} channel currents with subsequent membrane depolarization and activation of L-type Ca^{2+}, TRPV1 and TRPC6 channels to promote influx of Ca^{2+} [14–16,30,31]. 20-HETE mediates pressure-dependent myogenic cerebral arterial constriction, which is responsible for autoregulation of cerebral blood flow during increases in mean arterial pressure [8,13].

A previous study [32] suggested that a rise in $[Ca^{2+}]_i$ in astrocytes in rat and mouse brain slice preparations can induce constriction of adjacent cerebral arterioles and that this effect was attenuated by HET0016, an inhibitor of 20-HETE synthesis [32]. From these findings, the authors proposed that a rise in astrocyte $[Ca^{2+}]_i$ activates PLA_2-dependent AA release which is catalytically converted to 20-HETE by CYP 4A ω-hydroxylase in adjacent cerebral arteriolar smooth muscle to trigger vasoconstriction. However, no evidence was indicated if rat brain astrocytes express CYP 4A ω-hydroxylase or can metabolize AA to 20-HETE. The present study investigated whether cultures of neonatal rat brain astrocytes that are devoid of neurons express message and protein for CYP 4A isoforms and produce 20-HETE when incubated with AA. We have also examined contribution of 20-HETE to the actions of astrocyte conditioned media on reactivity of freshly isolated cerebral arterial myocytes, and the impact of inhibition of endogenous 20-HETE synthesis and application of exogenous 20-HETE on the open state probabilities of K_{Ca} channel currents recorded from cell-attached patches of cultured rat brain astrocytes. The present findings demonstrate that rat brain astrocytes express CYP 4A ω-hydroxylase message and protein, produce and release 20-HETE that could function as endogenous inhibitor of astrocyte K_{Ca} channel currents and influence the tone of cerebral arterial myocytes.

2. Methods

The animal protocols used in this study were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

2.1. Cell culture

Sprague Dawley rat pups of 2–3 days of age were anesthetized with diethyl-ether, decapitated and the brain removed for preparation of astrocyte cultures as previously described [5]. Briefly, the brain was dissected free of meninges, and the cerebral cortices and hippocampus were isolated and cut into small pieces, and transferred to sterile dish containing 20 U/ml papain (Worthington Biochemical Corp) and cysteine (0.15 mg/ml; Sigma) dissolved in Earle's balanced salt solution (Gibco BRL) and incubated at 37 °C for 40 min with gentle agitation. Digestion was stopped by washing three times with an astrocyte growth medium containing DMEM, 10% fetal bovine serum (FBS), 25 units/ml penicillin, 25 μg/ml streptomycin and 0.1% gentamicin (Invitrogen, Carlsbad, CA). The tissue was then dissociated by trituration with flame-narrowed Pasteur pipette and cell suspension was diluted with feeding medium and seeded at an initial density of approximately 2×10^5 cells per square centimeter. The cells were incubated at 37 °C in a 95%/5% mixture of atmospheric air and CO_2. The medium was changed after 2 days and subsequently twice a week. Confluent monolayers of brain astrocytes were studied.

2.2. CYP 4A mRNA analysis

Real-time PCR was used to measure the expression of *CYP 4A2* and *4A3* mRNA in primary cultures of rat brain astrocytes. Confluent cultured astrocytes were serum starved for 12 h in medium containing low-glucose (1 g/l) DMEM, 1% horse serum, 25 units/ml penicillin and 25 μg/ml streptomycin (Invitrogen Corporation, Carlsbad, CA). The cultured astrocytes were rinsed twice with DPBS (Dulbecco's Phosphate Buffered Saline), lysed on ice with a R-I buffer (Axygen Biosciences, Union City, CA, USA), which nullifies endogenous RNase activity, and collected in 1.5 ml tubes. Total RNA was isolated using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences). The samples were treated with DNase I (ThermoFisher Scientific Incorporated, Waltham, MA) to prevent contamination of the RNA with genomic DNA. RNA integrity was checked by electrophoresis on a formaldehyde agarose gel and the concentration of RNA was quantified using a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific Incorporated). For each sample, 1 μg of RNA was reverse transcribed in a 20 μl reaction using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). For RT-PCR, 5 ng of the cDNA was amplified in a 25 μl reaction containing iQ SYBR Green Supermix (Bio-Rad Laboratories) using an iCycler thermal cycler and iQ5 detection module (Bio-Rad Laboratories). After denaturing of the samples for 3 min at 95 °C, the reactions were amplified for 40 cycles for 30 s at 95 °C, 30 s at the annealing temperature (56 °C for *Polr2* and 56.5 °C for CYP 4A2/3). The primers were obtained from Operon Biotechnologies (Huntsville, AL). The primer sequences for *Polr2 were*: forward 5′ctgatgcgggtgctgagtcagaagg3′ and reverse 5′gcggttgaccccatgacgagtg3′. The sequences for the CYP 4A2/3 primers were taken from a previous publication [33]. The forward primer was: 5′ GTC CCC ATG CCA AGA CTT GT 3′ and the reverse primer was 5′ GTC TGG AGT AAA AGC TTT GGA GCT 3′. The linearity of amplification for both primer sets was verified by analysis of serially diluted cDNA. Product specificity was confirmed by melt curve analysis and agarose gel electrophoresis. Sequence analysis of the product formed could not differentiate between 4A2 vs. 4A3. Recent data indicates that the most likely CYP 4A isoform for the formation of 20-HETE in rat cerebral arteries is 4A3 [34,35]. Relative quantification of expression was determined by measuring the threshold cycle (Ct) values of each sample using the $2^{-\Delta\Delta Ct}$ method [36]. Relative abundance of CYP 4A2/3 was normalized to Polr2 mRNA expression. Data are presented as mean ± SEM.